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Antioxidant and Antibiotic-modulatory Potential of *Teucrium polium* Ethanolic Extract Against Genetically-modified Antibiotic-resistant *E. coli* Strains

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ABSTRACT

Background and Aim: The ethanolic extract of *Teucrium (T.) polium* (TPE) was investigated for its phenolic and flavonoid content, antioxidant activity, and potential to modulate antibiotic efficacy against antibiotic-resistant *E. coli* strains.

Materials and Methods: Antioxidant properties of TPE were evaluated by chemical-based tests application (total phenolic and flavonoid content, anti-radical activity in 1,1-diphenyl-2-picrylhydrazyl (DPPH) test). Antibacterial properties of TPE alone and in combination with antibiotics, as well as their effect on proton flux across the bacterial cell membrane were assessed.

Results: TPE demonstrated significant antioxidant activity (IC_{50} = 73.89 µg/mL) and contained high levels of phenolic (181.7 ± 2.1 mg GAE/g (gallic acid equivalents)) and flavonoid (95.4 ± 3.1 mg QE/g (quercetin equivalents)) compounds. Although TPE exhibited no direct antibacterial effects against the tested microorganisms, it significantly enhanced the efficacy of ampicillin and kanamycin against these strains by decreasing minimum inhibitory concentration (MIC) of ampicillin and kanamycin twice. TPE reduced MIC values of both antibiotics and affected bacterial growth kinetics by reducing growth rates and prolonging lag phases in resistant *E. coli* strains. Furthermore, TPE influenced membrane-associated properties, specifically reducing proton flux in bacteria, which likely contributed to its modulatory effects. Moreover, in case of the kanamycin-resistant *E. coli* pARG-25 strain, proton flux was suppressed up to 50% under TPE influence and almost 70% with kanamycin-TPE combination.

Conclusion: These findings demonstrated *T. polium* ethanolic extract as an effective antibiotic resistance modulator, offering a potential adjuvant therapy to combat bacterial resistance. Future research should be focused on identifying the specific bioactive compounds responsible for these effects and evaluating their therapeutic applicability.

Keywords: Antibiotic Resistance, Antioxidant, *E. coli,* Ethanolic Extract, Plant-derived Compound, Proton Flux, *Teucrium polium*

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1. Introduction

According to the World Health Organization (WHO), antibiotic resistance is one of the greatest threats to global health, food safety and development, causing an estimated 700,000 deaths annually **(1)**. Phytochemicals have great potential as a mean of combating antibiotic-resistant bacterial strains. Plant-derived substances can serve as new sources of antimicrobial agents due to their direct antimicrobial activity or possible synergistic interactions with antimicrobial agents, which can increase the susceptibility of bacteria to antibiotics **(2)**. Plant extracts or their individual components offer unlimited possibilities for controlling bacterial growth due to their biochemical diversity **(3)**. Nowadays, in the context of widespread antibiotic resistance, plants could serve as one of the most promising sources of new antimicrobial compounds.

Lamiaceae is one of the largest families of flowering plants, comprising about 250 genera and over 7,000 species. Most of the plants in this family are aromatic and therefore important sources of essential oils **(4)**. Species belonging to this family are widely used as spices as well as in folk medicine **(5)**. The genus *Teucrium*, which includes over 300 species, is the largest one in the Lamiaceae family. *Teucrium (T.) polium* produces tannins, terpenoids, saponins, flavonoids, sterols, β-caryophyllene, diterpenoids, caryophyllene oxide, asparagine, and resinous substances. These are used for the treatment of different diseases **(6)**.

While previous studies have highlighted the therapeutic potential of *T. polium*, its specific mechanisms of action against antibiotic-resistant bacteria and its antibiotic-modulating effects remain underexplored. The antibiotic-modulating activity of plant extracts, including *T. polium*, is often attributed to their ability to inhibit bacterial efflux pumps, disrupt membrane integrity, and enhance antibiotic uptake by altering permeability **(7, 8)**. Plant extracts can exhibit antibiotic-modulating activity by influencing bacterial metabolism, and growth rate and suppressing ionic fluxes, such as proton fluxes **(9)**.

The present research aimed to determine the concentration of total phenols and flavonoids in *T. polium* ethanolic extract and, most importantly, to evaluate its antibiotic-modulatory activity mechanisms in some gene-modified antibiotic-resistant bacteria.

Although several studies have examined the antibacterial potential of *T. polium* and its phenolic and flavonoid composition, the plant material used in our study was harvested from another geographical region (Armenia) that may result in a distinct chemical composition. The novelty of our work lies in membrane-related parameters investigation, particularly the antibiotic-resistant *E. coli* membrane proton flux that interestingly decreased in our study **(10, 11)**.

2. Materials and Methods

2.1 Plant Samples

Teucrium polium plant samples were collected in Tavush province of Armenia (v. Aygedzor), at the elevation of about 700-800 m above the sea level. Plant samples were dried at room temperature (RT) in the shade, grounded, and stored in freezer (-18 to -20°C) until use. The plant was identified at the Department of Botany and Mycology, Faculty of Biology, Yerevan State University, Yerevan, Armenia. Dried plant samples were deposited at the Herbarium of the same Department and are available upon the reasonable request.

2.2 Extraction

Plant material was extracted using 70% ethanol (10 gr of dried plant material per 10-15 mL ethanol). The extraction was performed at RT, under agitation, using maceration technique. The resulting extract was centrifuged at 5000 rpm for 10 min. The supernatant was stored, and the precipitate was subjected to the same operation 4 more times. The supernatant of all stages were combined and dried at RT. The combined dry material was collected in Eppendorf tubes, weighed, and stored in freezer (-18 to -20°C) for further studies.

2.3 Bacterial Strains and Growth Conditions

During this study, different Gram-positive (*Bacillus subtilis* WT-A, *Staphylococcus aureus* MDC 5233, *Enterococcus hirae* ATCC 9790) and Gram-negative (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* MDC1754, *E. coli* K12, ampicillin-resistant *E. coli* DH5α-pUC18 and kanamycin-resistant *E. coli* pARG-25) bacteria were used as test microorganisms.

All used strains, including *E. coli* DH5α-pUC18 and *E. coli* pARG-25 strains, were provided by the Microbial Depository Center at the Armbiotechnology Scientific and Production Center, Yerevan, Armenia. The *E. coli* pARG-25 strain contains the high-copy cloning plasmid pARG-25 (KanR cassette), maintained in *E. coli* DH5α cells, which provides kanamycin resistance. The *E. coli* DH5α-pUC18 strain carries a pUC18 plasmid vector (AmpR cassete) constructed using the ampicillin resistance gene and pMB1 origin of replication from pBR322 **(12)**.

Bacteria were grown in a liquid Modified Peptone-Glycose broth medium (20 gr/L peptone, 2 gr/L glucose, 5 gr/L NaCl, 2 gr/L K_2HPO_4) at 37°C and pH 7.5 and incubated for 18-20 hr until reaching the stationary phase. The same medium was used for the disk-diffusion assay, but agar (1.7% w/v) was added to solidify.

2.4 Determination of Radical Scavenging Activity

The free radical scavenging potential of *T. polium* extract (TPE) was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Catechin was used as positive control. The sample solution contained 125 µL (1 mM) DPPH, 375 µL ethanol and 500 µL test-solution (extract or catechin with different concentrations (1000 - 10 µg/mL). The test solution was replaced by ethanol in the negative control sample. The absorbance was measured at 517 nm wavelength using a spectrophotometer Genesys 10S UV-Vis (Thermo Scientific, USA).

The radical scavenging activity was calculated using the following formula:

Radical scavenging activity (%) = Ac – As / Ac × 100,

Where Ac is the absorbance of the negative control, and as is the sample absorbance.

Data were presented as the extract IC_{50} value, which is the concentration of investigated samples required to decrease DPPH absorbance at 517 nm by 50% **(13)**. The IC_{50} value was calculated by non-linear scatter chart with trendline inhibition (Y-axis) vs concentration (X-axis) using Microsoft Excel.

2.5 Determination of Total Phenolic and Flavonoid Content of *T. polium* Extract

The quantitative determination of phenolic compounds was performed using the Folin-Ciocalteu assay with slight modifications **(14)**. Measurements were performed at 765 nm. Values were expressed in gallic acid equivalents in 1 gr of extract (GAE/g) **(15)**.

The total flavonoid content of the extract was assessed by employing an $AlCl_3$ colourimetric assay **(16)** using the calibration curve generated for quercetin (0–250 µg/mL). The results were expressed as mg of quercetin equivalents per 1 mg of extract (mg QE/g). Measurements were performed at 415 nm using a UV-VIS spectrophotometer (GENESYS 10S, Thermo Scientific, USA).

2.6 Investigation of Antimicrobial Activity of *T. polium* Extract

The antibacterial activity of TPE was evaluated using disk diffusion method at concentrations ranging from 0.125 to 1 mg/mL, prepared in two-fold serial dilutions (against both selected Gram-positive and Gram-negative bacteria). The two lower concentrations (125 and 250 µg/mL), which exhibited no inhibitory activity, were selected for the subsequent evaluation of antibiotic-modulating effects. Ethanol (70%) was used as negative control and the corresponding antibiotics (kanamycin (50 µg/mL), and ampicillin (25 µg/mL)) were tested as positive controls.

2.7 Determination of Antibiotic Modulatory Activity of *T. polium* Extract

The antibiotic modulatory activity of *T. polium* extract was explored by determining the minimal inhibitory concentrations of antibiotics (kanamycin and ampicillin) in the presence and absence of TPE at non-inhibitory concentrations. The decrease in MIC of antibiotics in the presence of plant extract shows its antibiotic modulatory activity. To calculate the modulation factor (MF), which indicates the synergistic or modulating interactions, the following formula was used:

$MF = MIC_{antibiotic}/MIC_{antibiotic+extract}$.

MF ≥2 indicates the occurrence of synergistic interactions, n = 1 means no impact **(9)**.

2.8 Determination of Growth Kinetics Under *T. polium* Extract Influence

The growth kinetics assay was employed to understand the pattern of bacterial growth under the influence of TPE, antibiotics, and antibiotic - TPE combination. New formed *E. coli* colonies were isolated from peptone agar plates and transferred to a liquid peptone medium (pH 7.5) followed by incubation for 24 hr at 37°C on 130 rpm/min shaker. The growth parameters of TPE were estimated at nontoxic concentration (0.125 mg/mL). Bacterial growth curves were determined by measuring the turbidity of samples containing bacteria at 565 nm every 30 min exploiting a McFarland DEN-1B densitometer (Biosan, Latvia) **(17, 18)** following the change in optical density (OD) of the culture relative to the medium every 30 min. The specific growth rate (µ) and the mean generation time (g) were determined by the following formulas:

$$\mu = \frac{\ln N2 - \ln N1}{t2 - t1}$$

$$g = \frac{\ln 2}{\mu}$$

Where N_2, N_1 are the optical densities (OD_{600}) of the bacterial culture at time points, and t_2 - t_1 = time interval in hours, respectively. The values were obtained during the exponential growth phase. The specific growth rate (µ) was determined as the ratio of difference in logarithmic values of doubled optical densities to the doubling time and was expressed in

units of h^{-1}. The duration of the lag phase was determined by the expression ln2/μ.

2.9 Investigation of Proton Flux Rate

Bacterial cells (in the late exponential growth phase) were collected by centrifugation at 3500 x g for 10 min using a Sorvall LYNX 6000 Superspeed Centrifuge (Thermo Scientific, USA). After the centrifugation, the cell pellet was exposed to two times washing with distilled water, and the third centrifugation with 150 mM Tris–HCl (pH 7.5) buffer solution (containing 0.4 mM $MgSO_4$, 1 mM NaCl, and 1 mM KCl). The H^+ flux rate was determined by the whole cells in the presence of glucose (2g/L) as described **(19)**. TPE (125 μg/mL) was added to the cell suspension, and the pH changes were recorded each 30 second using a pH meter (Hanna Edge pH Meter, Hanna Instruments, USA) equipped with a selective HI11310 Glass Body Refillable pH Electrode. The same protocol was applied for the cells treated with ATPase inhibitor N, N'-dicyclohexylcarbodiimide (DCCD, 0.1 mM). The proton flux rate was calculated as the negative logarithm of the proton concentration and expressed as mmol H^+/min per 10^8 cells per mL. For the control strain, both antibiotic variants—kanamycin and ampicillin—were used to compare changes with the resistant strains. For the antibiotic-resistant strains, only the relevant antibiotic was used: kanamycin for *E. coli* pARG-25 and ampicillin for *E. coli* DH5α-pUC18. Negative controls containing ethanol (at the same final concentration used to dissolve the extract) were also included to assess any potential effects of the solvent on membrane proton flux. As expected, ethanol at a final concentration below 1% had no measurable effect on the proton flux rate.

2.10 Chemicals and Reagents

Ethanol (POCH S. A., Lot # 1156/11/21) was purchased from Gliwice, Poland, DPPH (Lot #STBB0828V), Folin-Ciocalteu reagent (Lot # BCBX8424), gallic acid (Lot # MKCX6057), kanamycin sulfate (Lot # 066M4019V), ampicillin sodium salt (Lot # BCBW1243), catechin-amino2-(hydroxymethyl)-1,3-propanediol (Trizma-Base) (Lot # BCBF0735V), agar (Lot # BCCJ3606), peptone (Lot # BCCN1582), quercetin (Lot # 11015TG), and DCCD (Lot # WXBF5616V were all prepared from SigmaAldrich, Taufkirchen, Germany. NaCl (Lot # 23C154132), K_2HPO_4 (Lot # 24G194117), and glucose (Lot # 23E1556711), Tris (Lot # 22J0444105) were provided by VWR, Leuven, Belgium.

2.11 Statistical Analysis

The results were presented as means ± standard deviation (SD) of three independent repetitions. The statistical significance of the results was examined using one-way ANOVA followed by Dunnett's test or two-way ANOVA and Tukey's multiple comparisons tests. The statistical analyses were performed using GraphPad Prism 8.0.2 software (GraphPad Software, Inc., San Diego, CA, USA) and a P-value less than 0.05 was considered significant.

3. Results

3.1 Radical Scavenging Activity Result

The DPPH assay showed that TPE possesses high anti-radical activity: the IC_{50} value was determined to be 73.89 μg/mL ($y = 0.1545x + 4.335$, $R^2 = 0.99$). For the positive control (catechin) this parameter value was calculated to be 13.08 ± 0.035 μg/mL ($y = 3.4343x + 5.0693$, $R^2 = 0.99$).

3.2 Total Phenolic and Flavonoid Content

The amount of total phenols in the ethanolic extract of *T. polium* was determined to be 181.7 ± 2.1 mg of GAE/g ($R^2 = 0.9905$). The total flavonoid content of the *T. polium* extract was determined to be 95.4 ± 3.1 mg of QE/g ($R^2 = 1$).

3.3 Antimicrobial Activity Results

Preliminary studies revealed that TPE did not exhibit antibacterial activity at concentrations up to 1 mg/mL on tested Gram-positive and Gram-negative microorganisms. However, the extract exhibited antibiotic-modulating properties.

3.4 Antibiotic modulatory activity Results

When evaluated against ampicillin-resistant *E. coli* DH5α-pUC18 and kanamycin-resistant *E. coli* pARG-25 strains, the compound lowered the MIC of ampicillin and kanamycin by 50%. This reduction suggests a synergistic interaction, as indicated by MF≥2, which is a commonly accepted threshold for the synergy in antimicrobial combination studies. The observed effect implies that TPE enhances the antibacterial activity of these antibiotics, potentially by interfering with resistance mechanisms or increasing antibiotic uptake (Figure 1).

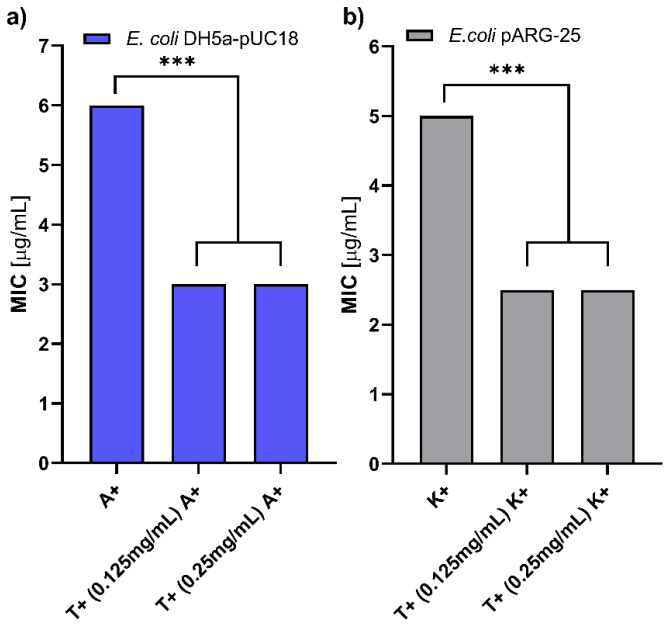

Figure 1. Modulatory effect of *Teucrium polium* ethanol extract (TPE) on the minimum inhibitory concentrations (MICs) of ampicillin (a) and kanamycin (b) against gene-modified resistant *E. coli* strains. TPE reduced MIC values at 0.125 mg/mL and 0.25 mg/mL concentrations, respectively in a statistically significant manner (***: $P<0.001$). Data represent means ± SD from three independent experiments performed in triplicate. Experimental conditions: A+ (treated with ampicillin), K+ (treated with kanamycin), T+K+/T+A+ (TPE + antibiotics), (K = kanamycin, A = ampicillin).

3.5 Growth Kinetics Results

Further the influence of TPE was evaluated on bacterial growth parameters. The specific growth rate (µ) of *E. coli* K12 decreased by 1.3-fold following TPE treatment, declining from 1.098 to 0.812. This reduction was accompanied by an increase in mean generation time, indicating a prolonged adaptation period (Figure 2a). In the ampicillin-resistant *E. coli* DH5α-pUC18 strain, the difference in growth rates between antibiotic-alone and antibiotic-TPE combination treatments was minimal (µ values: 0.357 vs. 0.362), suggesting that TPE had little impact on bacterial growth in the presence of ampicillin (Figure 2b). Additionally, TPE alone did not significantly alter the growth parameters.

For kanamycin-resistant *E. coli* pARG-25 strain, TPE treatment resulted in a 30% reduction in bacterial growth rate (from $\mu = 0.78$ to $\mu = 0.55$). When combined with kanamycin, TPE also extended the lag phase by 40%, suggesting a delay in bacterial adaptation to antibiotic stress (Figure 2c). These findings indicate that TPE modulates bacterial adaptation and growth dynamics, particularly in the presence of kanamycin, potentially by enhancing antibiotic susceptibility or affecting bacterial metabolism.

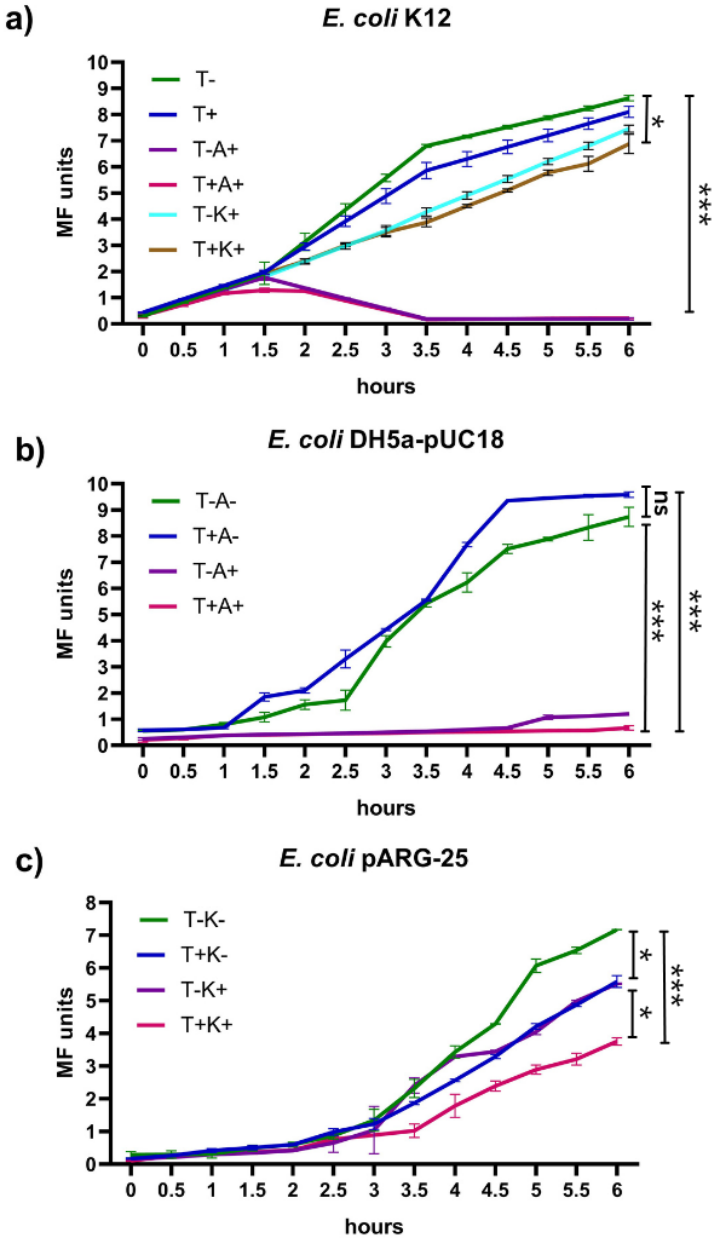

Figure 2. The specific growth rates of *E. coli* K12 (a), *E. coli* DH5α-pUC18 (b), and *E. coli* pARG-25 (c) strains under the influence of TPE at nontoxic concentration (0.125 mg/mL). Data represent means ± SD from three independent experiments performed in triplicate (ns: not significant; *: $P<0.05$, ***: $P<0.001$). Experimental conditions: T- (control, no treatment), T+ (treated with TPE), T-A-/T-K- (no TPE, no antibiotics), T+A-/T+K- (TPE only), T+K+/T+A+ (TPE + antibiotics), (K = kanamycin, A = ampicillin).

2.6 Proton Flux Rates Decreased

Investigations into membrane-associated properties revealed a 1.26-fold decrease in proton flux rate for *E. coli* K12 treated with TPE. No significant differences were observed between TPE-alone and antibiotic-TPE combination treatments for this strain (Figure 3a). In ampicillin-resistant *E. coli*, TPE reduced proton flux by 2.2-fold, with antibiotic-TPE combinations showing an additional 30% decline (Figure 3b). For kanamycin-resistant *E. coli* strain, proton flux decreased nearly 2-fold with TPE alone and 2.9-fold with the antibiotic-TPE combination (Figure 3c).

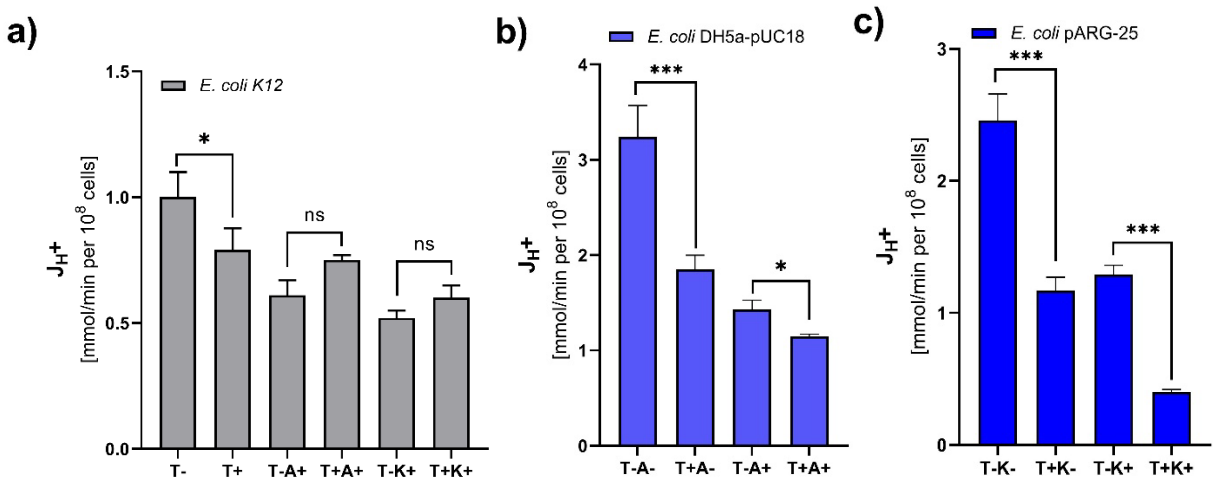

Figure 3. The total proton flux rate of membranes of *E. coli* K12 (a), *E. coli* DH5α-pUC18 (b), and *E. coli* pARG-25 (c) strains under the treatment of TPE and antibiotics. Data represent means ± SD from three independent experiments performed in triplicate (ns-not significant; *: *P*<0.05, ***: *P*<0.001). Experimental conditions: T- (control, no treatment), T+ (treated with TPE), T-A-/T-K- (no extract, no antibiotics), T+A-/T+K- (TPE only), T+K+/T+A+ (TPE + antibiotics), (K = kanamycin, A = ampicillin).

4. Discussion

In this work, we investigated the antimicrobial, and antibiotic modulatory properties of the ethanolic extract of *T. polium* (TPE) and tried to address the mechanisms of action. The modulation of the activity of different antibiotics possibly could be the result of the redox properties of the present compounds.

Initially we examined the potential of TPE to reduce free radicals. DPPH assay is a widely used and simple method for checking the ability of compounds to act as free radical scavengers or active reducing agents. Our investigations revealed that the anti-radical activity of TPE scavenges DPPH radicals *in vitro*. The strong antioxidant capacity of *T. polium* leaf extract was also reported in other research works **(20, 21)**. The high anti-radical activity of TPE observed in the DPPH assay aligns with its substantial phenolic and flavonoid content. Phenolic compounds are key contributors to the antioxidative properties of plant extracts, corroborating previous findings. Phenolic compounds are the most abundant plant secondary metabolites and have attracted increasing attention due to their wide range of biological activity including the antioxidant capacity and potential role in prevention of different oxidative stress-associated diseases, such as cancer, diabetes, neurodegenerative diseases, and aging process of the body **(22-24)**. The antioxidant properties of polyphenols can be explained by their ability to neutralize free radicals, and chelate redox-active iron and copper involved in the Fenton reaction **(25-28)**. Discrepancies in phenolic content reported by various studies highlight the influence of environmental and extraction factors on phytochemical profiles. Other studies report varying values depending on the region where the plant is grown. For instance, El Atki et al **(20)** reported 112.27 ± 4.75 mg GAE/g dry weight (DW), Qabaha et al **(29)** reported 155.2 ± 3.4 mg GAE/g DW, Ait Chaouche et al **(30)** reported 157.42 ± 4.12 mg GAE/g DW, and Timizar et al **(21)** reported 227.43 mg GAE/g DW. Concerning the content of flavonoids, Timizar et al **(21)** reported 20.78 mg QE/g, Ait Chaouche et al **(30)** reported 34.46 ± 2.24 mg QE/g, and Qabaha et al **(29)** reported 67.2 ± 1.5mg CA/g for TPE. Flavonoids are the most common and widely distributed group of plant phenolic substances. Total flavonoid concentration may provide indication of the relationship between anti-radical activity and the flavonoids content. Rather high total flavonoid content in TPE explains its high anti-radical and antioxidant activity. But the scavenging effect is not limited only to flavonoid compounds. The activity might also be related to the presence of different phenolic substances, volatile oils, carotenoids and vitamins **(31)**. These parameters could indicate the presence of possible antimicrobial activity of the examined extract.

Antibiotic resistance has become a major global health concern **(32)**. The need for new antimicrobial agents that can effectively combat resistant bacteria has increased tremendously **(33)**. As it was indicated above, the antimicrobial activity of TPE was determined using a range of Gram-positive (*B. subtilis* WT-A, *St. aureus* MDC 5233, *E. hirae* ATCC 9790) and Gram-negative (*E. coli* ATCC 25922, *S. typhimurium* MDC1754, *E. coli* K12, ampicillin-resistant *E. coli* DH5α-pUC18 and kanamycin-resistant *E. coli* pARG-25) bacteria. The obtained data indicated that TPE alone did not exhibit antibacterial properties against

any of the tested bacterial strains at concentrations up to 1 mg/mL. Plant extracts can possess direct antimicrobial action or act as antibiotic resistance-modifying agents and increase the efficiency of conventional antibiotics through different mechanisms **(34)**. Although the extract lacked direct antibacterial activity against tested strains at the investigated concentrations, its antibiotic-modulating properties were noteworthy. As it was mentioned, TPE effect in modulating the efficiency of ampicillin and kanamycin antibiotics was explored. The plant extract at nontoxic concentrations (NCTCs) for the test bacteria (0.125 mg/mL and 0.25 mg/mL) when combined with kanamycin and ampicillin decreased the MIC values of the antibiotics by two-fold against tested bacteria. The reduction of MIC values for ampicillin and kanamycin against resistant strains underscores the potential of TPE to enhance antibiotic efficacy. This property is particularly relevant in addressing antibiotic resistance challenges.

The observed modulation of bacterial growth parameters suggests that TPE affects bacterial adaptation mechanisms, potentially by targeting membrane-associated processes. For instance, the reduction in specific growth rate and the prolonged lag phase for resistant strains indicate altered metabolic activity under TPE influence. Notably, the lack of significant growth inhibition for *E. coli* K12 suggests strain-specific effects.

Further investigation of TPE effect on proton flux changes in the bacterial cell membrane are of importance. The proton flux studies provide insights into the mechanism underlying the antibiotic-modulatory effects of TPE. Reduced proton flux rates in antibiotic resistant strains (ampicillin-resistant *E. coli* DH5α-pUC18 and kanamycin-resistant *E. coli* pARG-25) treated with TPE, particularly in combination with antibiotics, suggest interference with membrane ion homeostasis. This disruption likely contributes to the enhanced antibiotic activity, as observed in the kanamycin-resistant strain. As reported by Dwivedi et al **(35)**, the clavine alkaloid, a plant-derived secondary metabolite, exhibits antibiotic-modulatory activity against tetracycline-resistant *E. coli*. It achieves this effect by inhibiting ATPase-dependent efflux pumps in the bacterial membrane **(35)**. In another case, certain plant extracts, such as those from *Azadirachta indica* and *Punica granatum*, exhibit antibiotic-modulatory activity by synergizing with antibiotics against antibiotic-resistant *E. coli*, likely through mechanisms involving efflux pump inhibition and disruption of membrane-associated processes, including the proton motive force **(36)**.

Overall, the findings highlight the potential of TPE as an adjuvant in antibiotic therapy. Future studies should aim to isolate and characterize active compounds responsible for these properties and investigate their mechanisms of action at molecular level. This study has limitations with no doubt. It would be desirable to continue studies on antibiotic-modulating activity and other biological parameters of the individual compounds present in the extract. However, due to technical limitations, it was not possible to isolate and characterize these specific compounds.

Validation plans

It is planned to continue the research under *in vivo* conditions, specifically by studying the effects of TPE on the intestinal microflora of animals. Future studies will also focus on further investigating the mechanisms of TPE action on antibiotic-resistant strains of *E. coli*.

5. Conclusion

The ethanolic extract of *T. polium* (TPE) demonstrated significant antioxidant activity alongside notable phenolic and flavonoid contents. These bioactive compounds likely contribute to the observed modulatory effects on antibiotic activity. While TPE alone did not exhibit direct antibacterial activity against the tested Gram-positive and Gram-negative microorganisms, it effectively enhanced the efficacy of ampicillin and kanamycin against resistant genetically-modified *E. coli* strains. When combined with antibiotics, the extract enhanced the efficacy of ampicillin and kanamycin against resistant bacterial strains, possibly by disrupting membrane-associated processes and interfering with proton flux. These findings suggest that TPE may serve as a promising adjuvant in combating antibiotic resistance. Further research is warranted to identify the active constituents and elucidate the underlying molecular mechanisms.

6. Declarations

6.1 Acknowledgment

Not applicable.

6.2 Ethical Considerations

This manuscript adheres to the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines for reporting observational research **(37)**.

6.3 Authors' Contributions

All authors contributed to the study conception and design. Anush Babayan and Silvard Tadevosyan performed the experiments; Naira Sahakyan, Silvard

Tadevosyan, and Anush Babayan drafted the first manuscript, were involved in the acquisition, analysis, and interpretation of data. All authors provided approval of the final submitted manuscript.

6.4 Conflict of Interests

The authors declare no conflict of interest.

6.5 Financial Support and Sponsorship

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6.6 Using Artificial Intelligence Tools (AI Tools)

Not applicable.

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